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CAN X-RAYS BE USED TO INCREASE LITTER SIZE IN THE MOUSE?

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Evidence that X-rays can sterilize gonads is abundant (Carter, Lyon, and Phillips, 1954; Casarett, 1956; Ingram, 1958; Jones, 1960; Mole, 1959; Nuzidin, Shapiro, Petrova and Kitaeva, 1955; Rugh, Deschner, and Grupp, 1960; Russell, 1958; Sanderson and Stearner, 1956), but there has also appeared in the literature some reports that low dose X-rays can stimulate ovulation, either to overcome sterility or to induce superovulation. Probably the first suggestion that X-rays could overcome amenorrhea or sterility in women was made by Fraenkel (1914). The next year Flatow made similar observations on the human female, but these were not reported until 1922. The idea was used in medical practice by Rubin (1926) and extensively by Kaplan (1938–1960), who published ten papers reporting direct ovarian X-radiation and occasionally supplementing it with X-radiation of the master reproductive gland, the pituitary, for some 3000 sterile women. He reported some 35% success in achieving pregnancies in women who had previously been diagnosed as being sterile, but multiple births were not significantly increased. He attributed his success solely to X-radiation. It was Asherman, however, who in 1952 suggested that simultaneous X-radiation of the ovaries and the anterior pituitary might be more effective than simply irradiating the ovary in overcoming sterility.

While no one can extrapolate data from another species to the human, and human data are rare and hard to come by in statistically significant numbers, it seemed in order to investigate this matter systematically on fairly large numbers of experimental mammals (mice) with varying exposures to whole-body X-rays, as well as at various times relating to the estrous cycle. Such a study is reported here.

MATERIALS AND METHODS

Sexually mature female (CF_1) mice, three to four months of age, were exposed to whole-body X-radiation to a maximum of 200r, and over 8000 offspring were counted. This exposure is far below the LD/50/30 (50% lethal dose in 30 days) of 610r X-rays for this strain of mice. The exposures were single and ranged from 10r to 200r after which unirradiated but sexually mature males were added either immediately or after one week (as designated). The delayed mating was used to allow one estrous cycle, which was producing a mature irradiated ovum, to pass so that the irradiated ovum from the succeeding estrus would be fertilized. Should superovulation be evident, this would mean stimulation of an earlier stage in oögensis.

The radiation factors were: Westinghouse, Kilovolt peak 250, milliamperes 15, filters 0.44 tin, 0.25 copper, 1 aluminum with half value layer of 2.37 mm copper, distance 171.2 cm target specimen distance, rate 6.23r/minute using a Regato collimator. For the 100r–200r levels of exposure the following factors were changed: filters 0.5 copper, +1 aluminum, half value layer 1.37 mm copper, distance 1 meter target specimen distance, and rate 32.5r/minute.

The females selected for the various estrous stages by vaginal smears were X-radiated twelve at a time in a screen-pie tray through which the rays were free to pass and in which the mice were free to move about within the radiation field. Calculations of dose rate were made with the same supporting materials used during exposure. Following exposure they were placed twelve to a large animal cage, and two males were added to each box, either immediately in one series or after one week's delay in the other series. This ratio of one male to six females is adequate to keep all females pregnant since their estrous cycles range over a five-day period and are rarely simultaneous. Even so any sexually mature male can impregnate three females in any 24-hour period. The second series was delayed so that the ova in the immediate process of meiosis were not involved in the experiment, but rather those öocytes being prepared for the succeeding ovulations were exposed and fertilized. In both series the control females for irradiation were selected at random, without respect to estrous phase.

All females were sacrificed and dissected at late pregnancy, usually day 18 or 19, just before expected birth. To allow delivery would introduce an erroneous set of data, since mice often will destroy their newborn especially when one or more happen to be malformed. In sacrificing before birth we are able to determine the number of implantations, resorptions, anomalies, and apparently normal fetuses. Since it has been suggested (Hahn, *et al.*, 1964–1972; Valentine and Hahn, 1971; Ward and Hahn, 1967; Feingold and Hahn, 1972, 1973) that the phase of the estrous cycle might somehow be related to different susceptibilities to induced superovulation, a second experiment was conducted to expose female mice to 10r at each of the major phases of the estrus cycle: estrus, diestrus, and metestrus. Metestrus, as used herein, includes both pro- and post-estrus. A group of randomly selected females that covered the gamut of the maturation stages (Womack and Bogart 1969) was used as non-exposed controls. Since Pozhidaev (1960) reported that a high exposure of 600 r induced superovulation in rats, it is quite possible that at our lower levels of exposure of female mice there may have been few, if any, pre-implantation deaths. However, Harvey and Chang (1964) stated that exposure of hamster ovarian oöcytes in estrus to X-rays from 25r to 200r caused some ova to develop micronuclei in direct proportion to X-ray exposure, and that these micronuclei might be the cause of some pre-implantation deaths. Therefore radiation during estrus led to reduced litter size. The data presented here include all fertilized ova that were implanted. We are primarily concerned with the size of the litters, and the anomalies that X-rays to the pre-fertilized ova could produce. In a recent paper (Rugh and Budd, 1975), it is shown that even with 500r whole-body exposure of mice, including both ovaries, there was no statistically significant change in litter size nor any evidence of congenital anomalies, when such ova were fertilized by normal non-irradiated sperm.

RESULTS

The average litter size and percentage resorption in sexually mature female (CF_1) mice exposed to various levels of X-radiation are shown in Table I. The

TABLE I

Litter size and resorptions in mice exposed to various levels of X-radiation with immediate mating.

Condition X-rays in r	Pregnancies	Number offspring	Average litter size*	Per cent resorptions
Controls	112	1179	10.5	1.5%
10r	46	439	9.54	3.4%
20r	11	119	10.8	1.0%
30r	47	505	10.8	4.0%
40r	222	2403	10.8	0.2%
50r	48	510	10.7	6.0%
100r	40	463	11.5	4.0%
200r	40	391	9.8	7.0%

* Note absence of any trend with increasing exposure dose.

females were selected without regard to their stage of estrous, and mature males were provided immediately after irradiation for mating on a continuous basis so as to be available to all females when they were in estrus. Table II shows average litter size for similar females exposed under similar conditions, but with a one week delay before mating.

Among litters resulting from immediate post-radiation mating, the variation in percent resorptions over exposure levels was minimal, while variation in average litter size was within the range of normal experience for these mice. Neither variable exhibited any trend over the exposure range. Among mice mated one week after exposure (Table II), the average litter size is lower in the control group than in the irradiated groups, but the magnitude of the difference is small and not statistically significant.

With the prior knowledge (Rugh and Budd, 1975) that even 500r to the female mouse would not produce anomalies, nor alter fertility when the oöcyte (as part of the body) is exposed to X-rays, the above data represent an exploratory venture to determine which lower exposure to X-rays might alter the resorption rate or even induce some degree of superovulation. Neither occurred to any significant degree.

TABLE II

Litter size in mice exposed to various levels of X-radiation with one week delay before mating.

Exposure in roentgens	Number of litters	Percent resorption	Litter size	
			Mean	Standard deviation
0r	16	1.5%	9.75	2.14
10r	21	2.4%	11.38	2.84
20r	19	4.8%	10.47	2.06
30r	23	6.5%	10.65	3.01
50r	19	3.6%	11.53	2.06
100r	14	6.0%	11.43	1.28

TABLE III

Exposure to 100r of X-radiation at various estrous stages followed by immediate mating.

Estrous stage	Number of litters	Code*	Percent resorptions	Litter size		
				Mean	Standard deviation	Standard error
Control**	18	a, b	1.0%	10.00	2.72	0.64
Estrus	31	a	2.8%	9.16	2.68	0.48
Diestrus	53	b, c	3.0%	11.15	2.58	0.35
Metestrus	25	c	1.8%	11.72	1.57	0.31

* Code: Means with different codes are significantly different when tested at the 5% probability level ($P \leq 0.05$). Means with the same code letter are not significantly different.

** Control: Females chosen at random without respect to estrous stage.

The results of exposure to 100r of X-irradiation at the various estrous stages followed by immediate mating are shown in Table III. No statistically significant difference in average litter size occurred between groups exposed at diestrus and metestrus, but both groups had slightly larger mean litter size than those exposed at estrus ($P \leq 0.05$). The unirradiated control group, which probably included all stages of estrous was intermediate in mean size of litter and not significantly different from the groups irradiated at either estrus or diestrus.

When the females were selected as to stage of estrous, with metestrus including both pro- and post-estrus, it was found that there was no statistically significant difference in litter size between those in estrus and the controls, which latter group included at random probably all stages of estrous. Likewise, when those in diestrus and metestrus were compared with the controls, there was no statistical difference in litter sizes. However, when those in diestrus or metestrus were compared with those in estrus, it was found that there was a statistically significant difference at the 5% (or less) probability level (*i.e.*, $P \leq 0.05$). Nevertheless one could hardly categorize the increases as evidence of superovulation.

DISCUSSION

Superovulation is presumed to mean stimulation to excess ovulations in the normal ovary. Over many years the CF_1 mouse has been found to produce an average of 10.5 mature ova from both ovaries at each estrus, so that superovulation would presume a statistically significant increase in this number. Superovulation does not guarantee that the ova are entirely normal, nor that they could give rise to normal and viable offspring. To stimulate an ovary to excess or premature ova production would require some catabolic enzyme acting on the follicular membranes (Rugh, 1935) thus releasing extra ova. Also, there might be some stimulation to the maturation process from the anterior pituitary growth promoting hormone which might result in gross and precocious enlargement of each ovarian follicle. There are, of course, gonadotropic hormones from the mouse pituitary (as in all mammalian pituitaries) which stimulate maturation and release of ova (Rugh, 1935; Austin, 1950) which can then be fertilized. The anti-sterility, or fertility hormones now in use with the human patients do just that, but such ova are probably perfectly normal since they are released by hormones normal to ovulation in the human female. The point here is that mere superovulation is no guarantee of normality, except probably when appropriate hormones are used to accelerate the normal processes (*e.g.*, Amphibia).

It was not until 1954 and 1956 that the Russells first reported controlled experiments on female mice wherein X-radiation of the ovaries did temporarily seem to stimulate them to superovulate. Pozhidaev (1960) claimed that 600r to rats increased their corpora lutea by 50%, and since 1964 Hahn and coauthors have published fourteen papers (1964–1972), suggesting that ionizing radiations do indeed induce superovulation in their chosen strain of mice. Their most recent papers were in 1973 (Hahn and Feingold; Feingold and Hahn).

There remains some confusion about the matter—for rats, mice, hamsters, and humans do not react in precisely the same manner to ovarian X-radiation, and superovulation following X-radiation has not always been achieved. Further, some evidence suggests initial stimulation with subsequent sterility (Rugh and Wolff, 1956, 1957; Rugh, 1964). It was also shown by Muramatsu, Suguhara, Tsuchiya and Okozowa in 1965 that chronic low-dose exposure of female rodents during their reproductive period would result in at least three successive generations showing increased mean litter size, but that ultimately sterility would set in. Mondorf and Faber (1968) stated that human females X-irradiated in childhood were slightly more fertile than nonirradiated females, and Meyer, Merz and Diamond (1969) made a very extensive analysis of human data from an epidemiological study of pelvimetry to find that among black women, the birth rate was 10 to 15% higher among those who had been X-rayed *in utero* than among matched controls (Meyer and Tonascia, 1973).

Various explanations have been offered for the experimental and clinical findings, largely relating to hormonal imbalance, effects on the female genital tract (Hahn and Feingold, 1972), production of micronuclei in the ova (Harvey and Chang, 1963), hyperemia of the ovary, and the production of mutations. In some cases it seemed that the same explanation could be used for opposite effects, namely superovulation (stimulation) or sterility.

In a recent study (Rugh and Budd, 1975), it was shown that whole-body X-ray exposure of sexually mature female mice at any time from 10r to 500r prior to their mating with normal males of the same strain caused no significant change either in litter size or anomaly production. Ova exposed to even 500r, (which is about 120r short of the LD/50/30 dose for this strain of mice) were not killed and F_1 offspring from such ova were fertile and did not produce anomalous fetuses. There was slight reduction in litter size when the exposures were 300r and 500r, but the litters were found to be almost within the normal size range. These results were explained as being due to the protective effect of the normal genes from the nonirradiated normal sperm used to fertilize the irradiated ova, thereby masking any mutations induced in the ova. Mole (1959), Mandl and Zuckerman (1961), and Lacassagne, Duplan, Marcovich, and Raynaud (1962) had shown that whole-body X-radiation of female mice did result in impairment of fertility.

For the above reasons, emphasis in this current study is placed upon the numbers of offspring, rather than on numbers of corpora lutea and possible pre-implantation deaths.

CF_1 female mice go through an estrous cycle every 4.5 to 5 days, producing from 1 to 17 mature ova with an average of about 10.5. Since females selected at random could be in any phase of estrous, it was proper to allow one X-radiated cycle to pass through and be discarded so as to determine whether X-rays would stimulate the ovary to excess ova production among the oöcytes immediately ready for the succeeding cycle. To do this there was a delay of one week between X-radiation and mating (Table II). Changes in the average litter size were not significantly different, hence no induced superovulation. There is no basis for suggesting that an embryonic or fetal loss may have balanced out some presumed superovulation. Suggestions have been made that the age or stage of the oöcytes might make a difference in their responses to ionizing radiations. Thus the study was made on X-radiating the various stages of estrous (Womack and Bogart, 1969; Rugh, 1968). The study included 255 pregnancies and 2750 offspring; and the evidence suggested a slight reduction in litter size when the female was in estrus, but no superovulation at any time.

Whole-body X-radiation naturally includes the anterior pituitary gland, known as the master gland for reproduction, which might have an indirect effect on ovulation. Whether exclusive exposure of this gland might induce superovulation has not been reported. It is located in mid-center of the brain and would be difficult to find in focal X-radiation. Of course this gland is included in every whole body X-radiation, as are both ovaries (Mateykö and Edelmann, 1954). The knowledge that the anterior pituitary is the master reproductive gland led gynecologists and obstetricians to prescribe simultaneous pituitary and ovarian X-radiation of human females hoping to help them overcome their sterility (Kaplan 1938–1960; Fullenlove, Haman and Williams, 1956; Morgan and Reyes, 1957; Rugh and Clugston, 1954a, b). The fact that female mice of this strain tended to tolerate whole-body X-radiation better than did the males suggested to Rugh and Clugston (1954a, b) that there might be estrous cycle variations in response related to hydration changes in the phases of that cycle. This was found to be true, with diestrus being the most radiosensitive and estrus the least. This difference was attributed more to changes in body hydration than to hormone production. Rugh and Wolff (1956, 1957) found that all female mice, randomly selected with respect to estrus and exposed to whole-body X-radiation could all be sterilized by 20r within eight weeks of exposure, but that in the interim normal-sized and viable litters were produced. Thus, not only were the immediate stages of estrous shown to be relatively insensitive, but earlier stages in oöcyte production were sensitive enough to be killed by 20r exposures. These conclusions have been supported by Peters (1961) and by Mole and Papworth (1966), for mice and rats, but not shown for hamsters or the human.

It has been found by Brambell and Parkes (1927) that whether there was local ovarian or whole-body X-ray exposure of female mice there resulted the same effect on reproductive performance. But it was also noted, as long ago as 1931 by Murray, that the histological structure of the mouse ovary was not a good indicator of the mouse's age. Nevertheless an exposure of as little as 54r to the ovaries did cause structural changes of a histopathological nature, appearing as early as one day after exposure (Murray, 1931). It was also suggested that structural sterility occurred after 150r in two days. This latter observation is not confirmed, except possibly for the appearance of micronuclei in the hamsters which (Harvey and Chang, 1963; 1964) may not be proof of degeneration.

Harvey and Chang (1963) found in the hamster that the most radio-sensitive period in oöcyte maturation was eight to twelve hours *before* ovulation, during final enlargement of the follicle. They shielded the ovaries and adnexa on one side during X-radiation, and compared ova production from the ovary of the other side in the same animal. They found (1964) no increase or decrease in ova production, only a delay in the mitotic divisions and appearance of micronuclei which they considered evidence of cellular breakdown. Using up to 200r to hamster ovarian oöcytes during their first meiotic division, and mating with normal males three hours later, resulted in no change in litter size or production, and so few anomalies (8 out of 350 hamsters) that they were not considered significant. Harvey and Chang found that *corpora lutea were not increased by X-radiations* of the randomly selected hamster females to 200–700r. Mandl (1963, pp. 119, 135, 136) first suggested that X-radiation of the ovary on one side of a rat would reduce ovulations in the exposed ovary but cause compensatory increase in ovulations from the other and unexposed ovary of the pair, attributing this "as due to non-specific damage to the ovary mediated through a reduction in the number of pre-existing large Graäfian follicles undergoing atresia."

In 1972 Byskov and Peters found that when Bagg female mice were exposed to 300r whole-body X-rays and immediately mated with sexually mature males, that the resulting F_1 offspring showed early signs of aging and varied fertility but without change in the number of oöcytes maturing. However the F_2 mice appeared to produce more young than the controls, suggesting that any superovulating effect was carried through the germ cells for two generations. Hahn and Feingold (1972) found a transitory unilateral reduction of ovulations following unilateral ovarian X-radiation in the rat and Feingold and Hahn (1973) changed the initial word in the title of a report from "transitory" to "prolonged". In this latter instance they shielded the right lower quadrant of the female rat during X-radiation (400r) and recorded reduction of ova shed from the irradiated ovary and reproductive activity for 120 days stating that "the long term combined reproductive output of both sides (both ovaries) was not significantly different from contemporary unirradiated controls" (page 53).

Human ovaries and sometimes the pituitaries of the same individual have been exposed to ionizing radiations with the hope of overcoming sterility, with apparently some success even though the cause and effect relationship could not be irrefutably proven (Kaplan, 1938–1960). Gynecologists have contended that an ovarian wedge section is more likely to achieve ovulation in a human ovary than are X-rays. (Dr. H. Speert, College of Physicians & Surgeons, personal communication.) Some rat and hamster experiments have been reported in which a degree of excess ovulation has been described as relating to dose and time of irradiation during the estrous cycle (Hahn, *et al.*, 1964–1971). Our experiments indicate that in the species used, ionizing radiations did not stimulate excess or superovulation and consequently litter production. There has been no assessment of any possible genetic effects, ultimate sterility, or even possible teratogenesis, in future generations. It is virtually impossible to extrapolate these findings to the human, particularly with comparable dosimetry.

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SUMMARY

1. The average litter size for normal CF_1 mice is close to 10.5, varying in these experiments from 9.75 to 11.73. Separate groups of controls were provided for each of the three major experiments.

2. Resorptions were found even among the control groups, with an average of 1.5%. Other anomalies were not encountered, even in offspring from irradiated ova. Resorptions following X-radiation of ovarian ova never exceeded 7%, which is still within the normal range.

3. Depending upon the experiments, sexually mature females were either taken at random with regard to their estrous cycle, or vaginal smears were made and the females categorized to select the particular phase of estrous cycle of concern.

4. Among litters from matings immediately after (whole-body) X-radiation from 10 to 200r, the variations in resorptions over exposure levels was minimal, while variation in average litter size was within the range of normal experience for these mice.

5. Even among mice mated one week after exposure the differences in litter sizes were small and the statistical significance tenuous.

6. It was found that even a maximum exposure of 200r altered neither the resorption rate or production of anomalies, nor induced any significant increase in litter size.

7. When the various phases of the estrous cycle were segregated for irradiation to 100r, it was found that those in estrus showed somewhat smaller mean litter size than those which were X-irradiated during diestrus or metestrus. However, when compared with the control group, which probably included all phases of the estrous cycle, there was no statistically significant change.

8. On the basis of this study of some 8000 offspring, there is no evidence of significant change in ova production following whole-body X-radiation from 10r to 200r of mature CF_1 female mice, and certainly no evidence of superovulation.

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